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(54) Title: GRAFTED COPOLYMERS AS GENE CARRIERS

(57) Abstract

A non-toxic, polymeric gene carrier used to deliver a selected nucleic acid into a host cell comprises a grafted copolymer having straight-chain amphiphilic side polymers grafted to a polycationic main polymer. The polycationic main polymer forms a complex with nucleic acids by electrostatic interaction. The amphiphilic side polymers reduce the cytoxicity of the polycation and enhance solubility. The molar proportion of side polymers may be adjusted to optimize transfection efficiency. Likewise, the ratio of nucleic acid to copolymer may be adjusted to optimize efficiency. The composition may be used in a method for transforming a host cell with a selected nucleic acid. Grafted copolymers of polyethylene glycol and poly-L-lysine (PEG-*g*-PLL) are particularly effective, achieving peak gene expression within 24 hours and persisting for at least 96 hours.

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GRAFTED COPOLYMERS AS GENE CARRIERS

BACKGROUND OF THE INVENTION

This invention relates to a gene carrier for use in connection with *in vivo* applications. More particularly, the invention relates to a non-toxic composition and a method for effectively delivering a selected nucleic acid into a host cell by forming an electrostatic complex of the nucleic acid with a cationic polymer. Graft copolymers of poly-L-lysine (PLL) and a polyoxyalkyl glycol are particularly effective.

Genes are very attractive candidates for therapeutic use in a variety of disease states due to their ability to produce bioactive proteins using the biosynthetic machinery provided by host cells. M.S. Wadhwa et al., 6 Bioconjugate Chemistry 283 (1995). There are many established protocols for transferring genes into cells, including calcium phosphate precipitation, electroporation, particle bombardment, liposomal delivery, viral-vector delivery, and receptor-mediated gene delivery. Id. Although all of these methods can be used for delivering genes into cultured mammalian cells, there are many difficulties in introducing genes into target cells *in vivo*. In particular, calcium phosphate and polycation precipitation are two techniques that are probably the most widespread in laboratory practices (PROFECTION Mammalian Transfection Systems, Technical Manual 2 (Promega Corp., 1990)); however, they are characterized by relatively low transfection efficiency. A.V. Kabanov & V.A. Kabanov, 6 Bioconjugate Chemistry 7 (1995). These two techniques appear ineffective for introducing RNA molecules into cells and cannot be used for transfection *in vivo*. Id.

Transfection methods using retroviral or adenoviral vectors, E. Gilboa et al., 4 Biotechniques 504 (1986); M.A. Rosenfeld et al., 252 Science 431 (1991), overcome some of these limitations. Retroviral vectors, in particular, have been successfully used for introducing exogenous genes into the genomes of actively dividing cells such that stable transformants are obtained. D.G. Miller et al., 10 Mol. Cell Biol. 4239 (1990). However, the method of using retroviral vectors for inserting genes into the host cell's genome depends on the viral infection pathway. Applying the retroviral method in human gene therapy raises serious concerns about possible recombination with endogenous viruses, oncogenic effects, and immunologic

reactions. A.V. Kabanov & V.A. Kabanov, 6 Bioconjugate Chemistry 7 (1995); H.M. Temin, 1 Human Gene Therapy 111 (1990). Such concerns have discouraged the use of viral vectors for human gene therapy. D.G. Miller et al., 10 Mol. Cell Biol. 4239 (1990).

On the other hand, non-viral gene delivery systems, such as cationic liposomes, H.M. Temin, 1 Human Gene Therapy 111 (1990), or PLL, G.Y. Wu & C.H. Wu, 263 J. Biol. Chem. 14621 (1988); E. Wagner et al., 87 Proc. Nat'l Acad. Sci. USA 3410 (1990); H. Farhood et al., 1111 Biochem. Biophys. Acta 239 (1992), have their own drawbacks. Even though they seem to be safe for human clinical use, typical non-viral systems provide low transfection efficiencies or cause precipitation of the nucleic acids. N.H. Caplen et al., 1 Nature Medicine 1 (1995). At present, the LIPOFECTIN (trademark of GIBCO/BRL) protocol seems to be most reliable in this category, J.H. Felgner et al., Enhanced Gene Delivery and Mechanism Studies with a Novel Series of Cationic Lipid Formulations, 269 J. Biol. Chem. 2550 (1994), but it bears the disadvantage of high cytotoxicity. H. Farhood et al., 1111 Biochim. Biophys. Acta 239 (1992).

In view of the foregoing, it will be appreciated that development of a gene delivery system that is both safe and efficient would be a significant advancement in the art.

BRIEF SUMMARY OF THE INVENTION

It is an object of the present invention to provide a composition and a method for delivering nucleic acids into cells.

It is also an object of the present invention to provide a composition and a method for gene delivery that are safe and efficient.

It is another object of the invention to provide an efficient, non-viral composition and a method of use thereof for delivering exogenous DNA or RNA to a target cell.

It is still another object of the invention to provide a method of delivering a gene into a target cell *in vitro*.

It is yet another object of the invention to provide a method of delivering a gene into a target cell *in vivo*.

These and other objects can be addressed by providing a composition for delivery of a selected nucleic acid into a target cell, wherein the composition is configured for forming an electrostatic complex with the selected nucleic acid, comprising a biocompatible graft copolymer of a cationic first polymer and an amphiphilic second polymer. Preferably, the first polymer is a member selected from the group consisting of poly(L-lysine), derivatives thereof, and mixtures thereof, and more preferably is poly(L-lysine). Preferably, the second polymer is a polyoxyalkyl glycol, such as those selected from the group consisting of polyethylene glycol homopolymers, polypropylene glycol homopolymers, alpha-substituted poly(oxyalkyl) glycols, poly(oxyalkyl) glycol copolymers and block copolymers, and activated derivatives thereof. Polyethylene glycol is particularly preferred. In a preferred embodiment of the invention, the graft copolymer comprises polyethylene glycol grafted to an ϵ-amino group of poly(L-lysine). The graft copolymer preferably comprises about 5 mole% to about 25 mole% of polyethylene glycol and, more preferably, about 10 mole% of polyethylene glycol.

In another aspect of the invention, a composition for delivery of a selected nucleic acid into a host cell comprises an electrostatic complex of a selected nucleic acid and a biocompatible graft copolymer comprising a cationic first polymer and an amphiphilic second polymer. In a preferred embodiment of the invention, the nucleic acid and the graft copolymer are present in a weight ratio of about 0.3 to 10. Preferably, the composition further comprises an effective amount of an anti-endosome functional agent, such as chloroquine. Such effective amount of chloroquine is preferably about 25-250 μM and more preferably about 75-150 μM.

In still another aspect of the invention, a method of transforming a host cell with a selected nucleic acid comprises contacting the host cell with an effective amount of an electrostatic complex comprising the selected nucleic acid and a biocompatible graft copolymer, wherein the biocompatible graft compolymer comprises a cationic first polymer and an amphiphilic second polymer; such that the host cell internalizes the selected nucleic acid.

In yet another aspect of the invention, a method of using a composition for delivering a selected nucleic acid to an individual comprises administering an effective amount of an electrostatic complex comprising the selected nucleic acid and a

biocompatible graft copolymer, comprising a cationic first polymer and an amphiphilic second polymer, such that the complex is systemically circulated and contacts a host cell such that the host cell internalizes the selected nucleic acid.

BRIEF DESCRIPTION OF THE SEVERAL VIEWS OF THE DRAWINGS

FIG. 1 is a schematic representation of complex formation between plasmid DNA and an illustrative gene delivery composition according to the present invention.

FIG. 2 shows an illustrative synthesis scheme of a grafted copolymer, PEG-*g*-PLL, according to the present invention.

FIG. 3 shows data from a fluorescence quenching assay: (□) PLL control; (◇) 5 mole% PEG-*g*-PLL; (○) 10 mole% PEG-*g*-PLL; (△) 25 mole% PEG-*g*-PLL.

FIG. 4 shows size (diameter) determinations by dynamic laser light scattering of pSV-β-gal (DNA), a complex of pSV-β-gal and PLL (PLL), a complex of pSV-β-gal and 5 mole% PEG-*g*-PLL (5 mol% PEG), a complex of pSV-β-gal and 10 mole% PEG-*g*-PLL (10 mol% PEG), and a complex of pSV-β-gal and 25 mole% PEG-*g*-PLL (25 mol% PEG).

FIG. 5 shows transfection efficiency for transfection of human liver carcinoma (HepG2) cells with pSV-β-gal using LIPOFECTIN reagent (Lipofectin), a PLL complex (PLL), a 5 mole% PEG-*g*-PLL complex (5 mol% PEG), a 10 mole% PEG-*g*-PLL complex (10 mol% PEG), and a 25 mole% PEG-*g*-PLL complex (25 mol% PEG).

FIG. 6 shows the transfection efficiency of human liver carcinoma (HepG2) cells as a function of the weight ratio of DNA to PEG-*g*-PLL.

FIG. 7 shows cell viability of human liver carcinoma HepG2 cells after transfection with pSV-β-gal using a control (media), LIPOFECTIN reagent (Lipofectin), PLL (PLL), a 5 mole% PEG-*g*-PLL complex (5 mol% PEG), a 10 mole% PEG-*g*-PLL complex (10 mol% PEG), and 25 mole% PEG-*g*-PLL (25 mol% PEG).

FIG. 8 shows transfection efficiency of human liver carcinoma HepG2 cells with pSV-β-gal using a 10 mole% PEG-*g*-PLL complex as determined 24, 48, 72, and 96 hours after transfection.

FIG. 9 shows the effect of chloroquine concentration on transfection of human liver carcinoma HepG2 cells with pSV-β-gal using a 10 mole% PEG-*g*-PLL complex.

DETAILED DESCRIPTION

Before the present compositions and methods of use thereof for gene delivery are disclosed and described, it is to be understood that this invention is not limited to the particular configurations, process steps, and materials disclosed herein as such configurations, process steps, and materials may vary somewhat. It is also to be understood that the terminology employed herein is used for the purpose of describing particular embodiments only and is not intended to be limiting since the scope of the present invention will be limited only by the appended claims and equivalents thereof.

It must be noted that, as used in this specification and the appended claims, the singular forms "a," "an," and "the" include plural referents unless the context clearly dictates otherwise. Thus, for example, reference to "an anti-endosome function agent" includes a mixture of two or more of such agents, reference to "an amphiphilic polymer" includes reference to one or more of such polymers, and reference to "a cationic polymer" includes reference to a mixture of two or more of such cationic polymers.

In describing and claiming the present invention, the following terminology will be used in accordance with the definitions set out below.

As used herein, "PEG-*g*-PLL" means a grafted copolymer wherein PEG or another poly(oxyalkyl)glycol is conjugated to an ϵ-amino group of a lysine residue of PLL.

As used herein, "x mol% PEG-*g*-PLL," where x is a number between 1 and 100, refers to PEG-*g*-PLL having x mole% of PEG. For example, 5 mol% PEG-*g*-PLL is PEG-*g*-PLL containing 5 mole% of PEG.

As used herein, "poly(oxyalkyl)glycol" refers to polyether glycol polymers that when grafted to PLL render the resulting composition non-toxic and water soluble. Each monomer portion of the polymer contains a carbon chain having up to about 5 carbon atoms. Preferred poly(oxyalkyl) glycols are selected from the group consisting of polyethylene glycol (PEG) homopolymers, polypropylene glycol homopolymers, alpha-substituted poly(oxyalkyl) glycols (such as methoxypolyethylene glycols or other suitable alkyl-substituted derivatives such as those containing C_1-C_4 alkyl groups), poly(oxyalkyl) glycol copolymers and block copolymers, and activated derivatives thereof. The poly(oxyalkyl) glycols used in the present invention preferably have a molecular weight of about 200 to about 50,000, and more preferably about 200 to about 20,000. An especially preferred poly(oxyalkyl) glycol is polyethylene glycol (PEG). PEG is preferred because it is inexpensive, approved by the U.S. Food and Drug Administration for administration to humans, and is resistant to eliciting an antibody response.

As used herein, "PLL" refers to poly(L-lysine), derivatives thereof, and mixtures thereof. The PLL preferably has a molecular weight in the range of about 200 to 50,000 and more preferable in the range of about 500 to 30,000.

As used herein, "effective amount" means an amount of a nucleic acid that is nontoxic but sufficient to provide the selected local or systemic effect and performance at a reasonable benefit/risk ratio attending any medical treatment.

As used herein, "administering" and similar terms mean delivering the complex formed by admixing the nucleic acid to be delivered with a gene carrier composition according to the present invention to the individual being treated such that the complex is capable of being circulated systemically to the parts of the body where the complex can contact the target cells. Thus, the composition is preferably administered to the individual by systemic administration, typically by subcutaneous, intramuscular, or intravenous administration, or intraperitoneal administration. Injectables for such use can be prepared in conventional forms, either as a liquid solution or suspension or in a solid form suitable for preparation as a solution or suspension in a liquid prior to injection, or as an emulsion. Suitable excipients include, for example, water, saline, dextrose, glycerol, ethanol, and the like; and if

desired, minor amounts of auxiliary substances such as wetting or emulsifying agents, buffers, and the like can be added.

Delivery of a nucleic acid, i.e. DNA and/or RNA, can be used to achieve expression of a polypeptide or to inhibit expression of a polypeptide through the use of an "antisense" nucleic acid, especially antisense RNA. As used herein, "polypeptide" means peptides of any length and includes proteins. The term "polypeptide" is used herein without any particular intended size limitation, unless a particular size is otherwise stated. Typical of polypeptides that can be expressed are those selected from the group consisting of oxytocin, vasopressin, adrenocorticotrophic hormone, epidermal growth factor, prolactin, luteinizing hormone releasing hormone, growth hormone, growth hormone releasing factor, insulin-like growth factors, insulin, erythropoietin, obesity protein such as leptin, somatostatin, glucagon, glucagon-like insulinotropic factors, parathyroid hormone, interferon, gastrin, interleukin-2 and other interleukins and lymphokines, tetragastrin, pentagastrin, urogastrin, secretin, calcitonin, enkephalins, endorphins, angiotensins, renin, bradykinin, bacitracins, polymixins, colistins, tyrocidin, gramicidins, and synthetic analogues, modifications, and pharmcologically active fragments thereof, monoclonal antibodies, and vaccines. This group is not to be considered limiting; the only limitation to the peptide or protein drug that may be expressed is functionality. Delivery of DNA and/or RNA is useful in gene therapy, vaccination, and any therapeutic situation in which a nucleic acid or a polypeptide should be administered in vivo. E.g., U.S. Patent No. 5,580,859, hereby incorporated by reference.

When the nucleic acid is DNA, it can be a DNA sequence that is itself non-replicating, but is inserted into a plasmid wherein the plasmid further comprises a replicator. The DNA may also contain a transcriptional promoter, such as the CMV IEP promoter, which is functional in humans. The DNA can also encode a polymerase for transcribing the DNA. Many expression vectors for expression of a cloned gene in a mammal are known in the art, and many such expression vectors are commercially available, for example, pEUK-C1 (Clontech, Palo Alto, Calif.). A gene of interest can be inserted into such an expression vector according to recombinant DNA technology well known in the art. E.g., J. Sambrook et al., Molecular Cloning: A Laboratory Manual (2d ed., 1989), hereby incorporated by reference.

The method can be used for treating a disease associated with a deficiency or absence or mutation of a specific polypeptide. In accordance with another aspect of the invention, the method provides for immunizing an individual , wherein such individual can be a human or an animal, comprising delivering a DNA and/or RNA to the individual wherein the DNA and/or RNA codes for an immunogenic translation product that elicits an immune response against the immunogen. The method can be used to elicit a humoral immune response, a cellular immune response, or a mixture thereof.

An illustrative method of forming the gene carriers according to the present invention is accomplished by grafting polymer monomethoxy polyethylene glycol (mPEG) to the ϵ-amino group of lysine of PLL. PEG, a straight-chain amphiphilic polymer has been used to modify several enzymes giving them longer half-lives *in vivo*. F.F. Davis et al., in 4 Enzyme Engineering 169 (1978); A. Abuchowski et al., 7 Cancer Biochem. Biophys.175 (1984). PEG has also been used to modify interleukin-2 to give it increased solubility and increased half-life *in vivo*. N.V. Katre et al., 84 Proc. Nat'l. Acad. Sci. USA 1487 (1987). The PEG-grafted PLL (PEG-*g*-PLL) provides a solubility increase when it forms an electrostatic complex with genes to be delivered (FIG. 1) because of the solubilizing effect of the PEG chains. A.V. Kabanov et al., 6 Bioconjugate Chemistry 639 (1995). The solubility increase induced by the added PEG chains favorably affects the transfection efficiency as compared to a plasmid DNA/PLL complex without increasing the cytotoxicity of PLL. Rather, the presence of the PEG chains also acts to reduce the cytotoxicity of the PLL base and improve transfection duration. The electrostatic complex is formed by the affinity of the positively-charged polymer (e.g. PLL) and the negatively-charged nucleic acid.

Example 1

Graft copolymers of PEG-*g*-PLL having PEG contents of 5 mol %, 10 mol % and 25 mol % were synthesized according to the procedure outlined in FIG. 2. Thionyl chloride ($SOCl_2$), triethylamine (TEA), dimethyl sulfoxide (DMSO), and methoxy polyethylene glycol ($mPEGOCH_2CH_2OH$; MW=550) were purchased from

Aldrich (Milwaukee, Wisconsin). PLL-hydrobromide (Repeating unit=120; MW=25,000) was purchased from Sigma (St. Louis, Missouri).

For example, synthesis of 5 mol % PEG-*g*-PLL was performed as follows: Methoxy PEG$OCH_2CH_2CO_2H$ was synthesized by alkylation of mPEGOCH_2CH_2OH with ethyl bromoacetate. Thionyl chloride (1.5 ml) was added to a round bottom flask containing 6 mg of mPEG$OCH_2CH_2CO_2H$ and refluxed for 40 min, followed by evaporation of thionyl chloride under vacuum. The resulting product was dissolved in 100 μl of DMSO, and added to 1 ml of DMSO solution containing 25 mg of PLL-hydrobromide and 160 μl of TEA while stirring at room temperature. After stirring for 30 min., 4 ml of deionized water was added and the reaction mixture was adjusted to pH 1 with 6 N HCl. Finally, the product was dialyzed against water and lyophilized. The content of PEG was determined by ^{1}H-NMR using D_2O as a solvent (not shown). A peak at about 3.5 ppm signified the presence of mPEG in the product.

Example 2

The plasmid pSV-β-gal (Promega Corp., Madison, Wisconsin; EMBL accession No. X65335) is a positive control vector for monitoring transfection efficiencies of mammalian cells. The pSV-β-gal plasmid contains a SV40 early promoter and enhancer sequence, transcription start sites, *E. coli lacZ* coding region encoding β-galactosidase, and SV40 small T antigen polyadenylation signals. SV40 early promoter and enhancer drive the transcription of the *lacZ* gene.

Equal amounts of plasmid DNA and ethidium bromide (1 μg each) were placed in 1 ml of buffer containing 20 mM Hepes (N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]) and 0.15 M NaCl, (pH 7.4). Various amounts of PEG-*g*-PLL prepared according to the procedure of Example 1 were then added to the plasmid DNA-ethidium bromide mixture. Fluorescence intensity of this mixture was measured with a spectrofluorometer (PC1, ISS Co., USA)(λ_{ex}=516 nm, λ_{em}=590 nm). FIG. 3 shows the results from this fluorescence quenching assay. These data indicate that PEG-*g*-PLL condenses up to 55% of the plasmid DNA. Almost no difference was observed in the plasmid DNA condensation ability of PLL compared to its PEG-modified derivatives.

Example 3

In this example, gel retardation assays were carried out to further determine whether PEG-*g*-PLL formed complexes with DNA. Mixtures of pSV-β-gal and PEG-*g*-PLL prepared as in Example 2 were fractionated by electrophoresis in a 1% agarose gel. After fractionation, the gel was stained with ethidium bromide (0.5 μg/ml) and illuminated on a UV illuminator. The movement of free plasmid DNA (not shown) was retarded as the amount of PEG-*g*-PLL for complex formation increased. These results indicate that PEG-*g*-PLL formed a stabale complex with plasmid DNA. Complete retardation was achieved at and above a 1:1 weight ratio of plasmid DNA:PEG-*g*-PLL.

Example 4

In this example, the sizes of plasmid DNA/PEG-*g*-PLL complexes prepared according to the procedure of Example 2 were measured by dynamic laser light scattering (Brookhaven BI-DS) at a 90° angle using a 1:3 weight ratio of plasmid DNA to PEG-*g*-PLL. Data were analyzed by a cumulative analysis. FIG. 4 shows the size of the plasmid alone and the size of complexes with various compounds. The complexes have an average diameter of about 300 nm.

Example 5

In this example, the efficacy of compositions according to the present invention for mediating *in vitro* transfection of mammalian cells was demonstrated. HepG2 cells (human liver carcinoma cells) were obtained from the American Type Culture Collection (Rockville, MD; ATCC accession No. 8065-HB). Transfection was performed using PEG-*g*-PLL in 96-well plates seeded at a cell density of 20 x 10^4 cells/ml. The plasmid pSV-β-gal DNA/PEG-*g*-PLL complex was prepared by mixing 1 μg of plasmid DNA and 3 μg of PEG-*g*-PLL in 100 μl of serum-free MEM medium and incubating it for 30 min at room temperature, followed by the addition of 10% (v/v) fetal bovine serum (Hyclone Laboratories, Logan, Utah) and 100 μM chloroquine (Sigma). Chloroquine, a cell permeant base, was used to partially neutralize acidic compartments of the cells and prevent the fusion of endosomes with lysosomes. P. Midoux et al., 21 Nucleic Acids Research 871-78 (1993). The

medium in each well of the 96-well plate was replaced by the transfection mixture. Cells were incubated for 4 hr in a tissue culture incubator (Napco Co.) at 37°C in 5% CO_2. Transfection mixtures were removed and fresh growth medium containing 10% fetal bovine serum was added to each well. Cells were incubated for an additional 44 hr in a tissue culture incubator at 37°C in 5% CO_2.

In situ X-gal (5-chloro-4-bromo-3-indolyl-β-D-galactopyranoside) staining of transfected cells was used for the detection of expressed β-galactosidase. J.R. Sanes et al., 5 EMBO J. 3133 (1986). Cells in each well of the 96-well plate were washed twice with 1X phosphate-buffered saline (PBS) and fixed by 100 μl of 0.25% (v/v) glutaraldehyde for 15 min at room temperature. Glutaraldehyde solution was then removed and cells were rinsed gently 3 times with 1X PBS. Next, 60 μl of X-gal solution (1 mg/ml X-gal, 2 mM $MgCl_2$, 5 mM $K_4Fe(CN)_6$ and 5 mM $K_3Fe(CN)_6$) was added, followed by incubation for 16 hr in a tissue culture incubator at 37°C. X-gal solution was the removed and cells were covered with 1X PBS. Cells expressing the β-galactosidase enzyme from transfection of pSV-β-gal plasmid were stained blue by X-gal and could be seen and counted under a microscope.

As shown in FIG. 5, transfection efficiency increased by up to 30-fold as the degree of PEG-modification increased. Of the three degrees of substitution tested, 10 mol % PEG-g-PLL showed the best transfection efficiency. The 25 mol% PEG-g-PLL showed significantly lower transfection efficiency. Thus, the optimal range of PEG-modification in PLL seems to be around 10 mol %. A commercially available transfection agent, LIPOFECTIN reagent (GIBCO BRL), showed slightly higher transfection efficiency than 10 mol % PEG-g-PLL in HepG2 cells. LIPOFECTIN reagent is a 1:1 (w/w) liposome formulation of the cationic lipid N-[1-(2,3-dioleyloxy)propyl]-n,n,n-trimethylammonium chloride (DOTMA), and dioleoyl phosphotidylethanolamine (DOPE) in membrane filtered water.

Example 6

In this example, different complexes were made with a constant amount of plasmid DNA (10 μg/ml) and increasing amounts of 10 mol% PEG-g-PLL (1-50 μg/ml). These complexes were then used to transfect HepG2 cells according to the procedure of Example 5. As shown in FIG. 6, transfection efficiency increased as the

weight ratio of PEG-*g*-PLL to plasmid DNA increased to 3:1. At higher ratios of PEG-*g*-PLL to plasmid DNA, transfection efficiency decreased.

Example 7

In this example, the cytotoxicity of PEG-*g*-PLL was determined. The percentage of living cells was determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide) assay of T. Mosman, 65 J. Immunol. Methods 55 (1983), hereby incorporated by reference.

At the end of the transfection experiment described in Example 5, the transfection mixture was replaced by fresh growth medium containing 26 μl of 2 mg/ml MTT solution. Plates were incubated for an additional 4 hr at 37°C in a tissue culture incubator, then MTT-containing medium was removed by aspiration, and 150 μl of DMSO was added to dissolve the formazan crystals formed by living cells. Absorbance was measured at 570 nm using a microplate reader (Model EL311, Bio-Tek instrument Co.), and the percentage of living cells was calculated from the following equation:

$$Cell\ viability\ (\%) = \frac{A_{570(sample)}}{A_{570(control)}} \times 100$$

As shown in FIG. 7, Both 5 mol %- and 10 mol %-modified PEG-*g*-PLL showed very mild cytotoxicity on HepG2 cells, whereas LIPOFECTIN reagent or PLL showed moderate to high cytotoxicity. Even though 25 mol %-substituted PEG-*g*-PLL resulted in a lower cell viability than 5 mol %- or 10 mol %-substituted PEG-*g*-PLL, its cytotoxicity was still lower than either LIPOFECTIN or PLL. Therefore, the compositions of the present invention are improvements over known substances with respect to cytotoxicity.

Example 8

In this example, the procedure of Example 7 was followed except that the cell viability assay was performed at 24, 48, 72, or 96 hours after transfection. Generally, a transfection assay is performed 48 hrs after transfection, but almost the same

transfection efficiency was obtained when the assay was performed after only a 24 hr incubation (FIG. 8). In the case of LIPOFECTIN reagent, β-galactosidase activity after a 24 hr incubation was half the β-galactosidase activity after a 48 hr incubation (data not shown). Further, the cells transfected by plasmid pSV-β-gal DNA/PEG-g-PLL mixture maintained its gene expression level up to 96 hr (FIG. 8).

Example 9

In this example, the role of chloroquine in transfection efficiency was determined. The procedure of Example 5 was followed except that chloroquine concentration was varied from 0-100 μM. FIG. 9 shows that chloroquine was not essential for successfully transfecting cells, however, chloroquine played an important role in increasing transfection efficiency. As the concentration of chloroquine increased up to 100 μM, the transfection efficiency increased up to 30-fold.

Example 10

In this example, a method of delivering a gene *in vivo* to an individual is illustrated. A nucleic acid encoding the leptin obesity protein, such as human leptin or a rat leptin cDNA, C. Guoxun et al., Disappearance of Body Fat in Normal Rats Induced by Adenovirus-mediated Leptin, 93 Proc. Nat'l Acad. Sci. USA 14795-99 (1996), or a mouse leptin cDNA, P. Muzzin et al., Correction of Obesity and Diabetes in Genetically Cloned Mice by Leptin Gene Therapy, 93 Proc. Nat'l Acad. Sci USA 14804-14808, both of which are hereby incorporated by reference, is known in the art. The mammalian expression vector, pEUK-C1 (Clontech, Palo Alto, Calif.) is designed for transient expression of cloned genes. This vector is a 4.9 kb plasmid comprising a pBR322 origin of replication and an ampicillin resistance marker for propagation in bacteria, and also comprising the SV40 origin of replication, SV40 late promoter, and SV40 late polyadenylation signal for replication and expression of a selected gene in a mammalian cell. Located between the SV40 late promoter and SV40 late polyadenylation signal is a multiple cloning site (MCS) of unique XhoI, XbaI, SmaI, SacI, and BamHI restriction sites. DNA fragments cloned into the MCS are transcribed as RNA from the SV40 late promoter and are translated from the first ATG codon in the cloned fragments. Transcripts of cloned DNA are spliced and

polyadenylated using the SV40 VPI processing signals. The leptin gene is cloned into the MCS of pEUK-C1 using techniques well known in the art, e.g. J. Sambrook et al., Molecular Cloning: A Laboratory Manual (2d ed., 1989). The resulting plasmid is delivered to a human or animal after incorporation into a complex according to the present invention illustrated in Example 2.

An effective amount of the resulting complex is systemically administered to an individual such that complex enters the bloodstream and contacts target cells. The target cells that are contacted by the complex take up the complex, thus internalizing the leptin DNA. The leptin DNA is then expressed in the recipient cell, resulting in a positive effect in treatment for obesity or diabetes.

The compositions of the present invention offer improved transfection ability than that obtained with PLL. The present compositions also demonstrate low cytotoxicity, early gene expression, and maintenance of the early gene expression level up to 96 hrs. These characteristics are advantages over prior art compounds such as LIPOFECTIN reagent.

CLAIMS

We claim:

1. A composition for delivery of a selected nucleic acid into a target cell, wherein said composition is configured for forming an electrostatic complex with said selected nucleic acid, comprising a biocompatible graft copolymer comprising a cationic first polymer and an amphiphilic second polymer.

2. The composition of claim 1 wherein said first polymer is a member selected from the group consisting of poly(L-lysine), derivatives thereof, and mixtures thereof.

3. The composition of claim 2 wherein said first polymer is poly(L-lysine).

4. The composition of claim 3 wherein said poly(L-lysine) has a molecular weight of about 200 to 50,000.

5. The composition of claim 1 wherein said second polymer is a polyoxyalkyl glycol.

6. The composition of claim 5 wherein said polyoxyalkyl glycol is a member selected from the group consisting of polyethylene glycol homopolymers, polypropylene glycol homopolymers, alpha-substituted poly(oxyalkyl) glycols, poly(oxyalkyl) glycol copolymers and block copolymers, and activated derivatives thereof.

7. The composition of claim 6 wherein said polyoxyalkyl glycol has a molecular weight of about 200 to about 50,000.

8. The composition of claim 7 wherein said polyoxyalkyl glycol has a molecular weight of about 200 to about 20,000.

9. The composition of claim 6 wherein said polyoxyalkyl glycol is polyethylene glycol.

10. The composition of claim 1 wherein said graft copolymer comprises polyethylene glycol grafted to an ϵ-amino group of poly(L-lysine).

11. The composition of claim 10 wherein said graft copolymer comprises about 5 mole% to about 25 mole% of polyethylene glycol.

12. The composition of claim 11 wherein said graft copolymer comprises about 10 mole% of polyethylene glycol.

13. A composition for delivery of a selected nucleic acid into a host cell comprising an electrostatic complex of the selected nucleic acid and a biocompatible graft copolymer comprising a cationic first polymer and an amphiphilic second polymer.

14. The composition of claim 13 wherein said first polymer is a member selected from the group consisting of poly(L-lysine), derivatives thereof, and mixtures thereof.

15. The composition of claim 14 wherein said first polymer is poly(L-lysine).

16. The composition of claim 15 wherein said poly(L-lysine) has a molecular weight of about 200 to 50,000.

17. The composition of claim 13 wherein said second polymer is a polyoxyalkyl glycol.

18. The composition of claim 17 wherein said polyoxyalkyl glycol is a member selected from the group consisting of polyethylene glycol homopolymers,

polypropylene glycol homopolymers, alpha-substituted poly(oxyalkyl) glycols, poly(oxyalkyl) glycol copolymers and block copolymers, and activated derivatives thereof.

19. The composition of claim 18 wherein said polyoxyalkyl glycol has a molecular weight of about 200 to about 50,000.

20. The composition of claim 19 wherein said polyoxyalkyl glycol has a molecular weight of about 200 to about 20,000.

21. The composition of claim 18 wherein said polyoxyalkyl glycol is polyethylene glycol.

22. The composition of claim 13 wherein said graft copolymer comprises polyethylene glycol grafted to an ϵ-amino group of poly(L-lysine).

23. The composition of claim 22 wherein said graft copolymer comprises about 5 mole% to about 25 mole% of polyethylene glycol.

24. The composition of claim 23 wherein said graft copolymer comprises about 10 mole% of polyethylene glycol.

25. The composition of claim 13 wherein said nucleic acid and said graft copolymer are present in a weight ratio of about 0.3 to 10.

26. The composition of claim 13 further comprising an effective amount of an anti-endosome function agent.

27. The composition of claim 26 wherein said anti-endosome function agent comprises an effective amount of chloroquine.

28. The composition of claim 27 wherein said effective amount of chloroquine is about 25-250 μM.

29. The composition of claim 28 wherein said effective amount of chloroquine is about 75-150 μM.

30. A method of transforming a host cell with a selected nucleic acid comprising contacting the host cell with an effective amount of an electrostatic complex comprising the selected nucleic acid and a biocompatible graft copolymer, wherein the biocompatible graft copolymer comprises a cationic first polymer and an amphiphilic second polymer, such that said host cell internalizes said selected nucleic acid.

31. The method of claim 30 wherein said first polymer is a member selected from the group consisting of poly(L-lysine), derivatives thereof, and mixtures thereof.

32. The method of claim 31 wherein said first polymer is poly(L-lysine).

33. The method of claim 32 wherein said poly(L-lysine) has a molecular weight of about 200 to 50,000.

34. The method of claim 30 wherein said second polymer is a polyoxyalkyl glycol.

35. The method of claim 34 wherein said polyoxyalkyl glycol is a member selected from the group consisting of polyethylene glycol homopolymers, polypropylene glycol homopolymers, alpha-substituted poly(oxyalkyl) glycols, poly(oxyalkyl) glycol copolymers and block copolymers, and activated derivatives thereof.

36. The method of claim 35 wherein said polyoxyalkyl glycol has a molecular weight of about 200 to about 50,000.

37. The method of claim 36 wherein said polyoxyalkyl glycol has a molecular weight of about 200 to about 20,000.

38. The method of claim 35 wherein said polyoxyalkyl glycol is polyethylene glycol.

39. The method of claim 30 wherein said graft copolymer comprises polyethylene glycol grafted to an ϵ-amino group of poly(L-lysine).

40. The method of claim 39 wherein said graft copolymer comprises about 5 mole% to about 25 mole% of polyethylene glycol.

41. The method of claim 40 wherein said graft copolymer comprises about 10 mole% of polyethylene glycol.

42. The method of claim 30 wherein said nucleic acid and said graft copolymer are present in a weight ratio of about 0.3 to 10.

43. The method of claim 30 further comprising an effective amount of an anti-endosome function agent.

44. The method of claim 43 wherein said anti-endosome function agent comprises an effective amount of chloroquine.

45. The method of claim 44 wherein said effective amount of chloroquine is about 25-250 μM.

46. The method of claim 45 wherein said effective amount of chloroquine is about 75-150 μM.

47. A method of using a composition for delivering a selected nucleic acid to an individual comprising administering an effective amount of an electrostatic complex comprising the selected nucleic acid and a biocompatible graft copolymer, comprising a cationic first polymer and an amphiphilic second polymer, such that the complex is systemically circulated and contacts a host cell such that the host cell internalizes the selected nucleic acid.

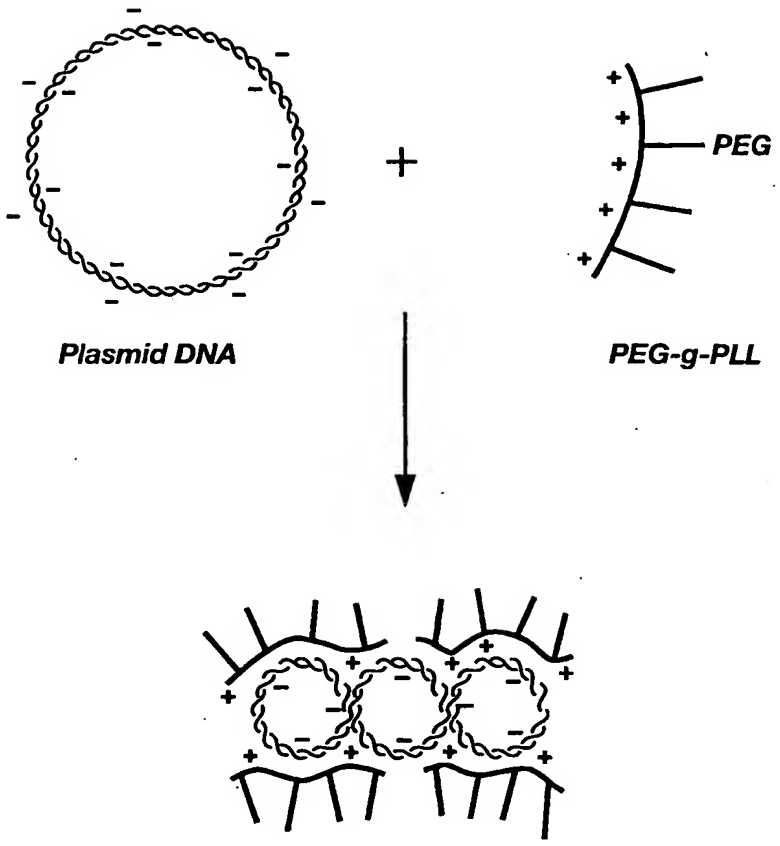

Fig. 1

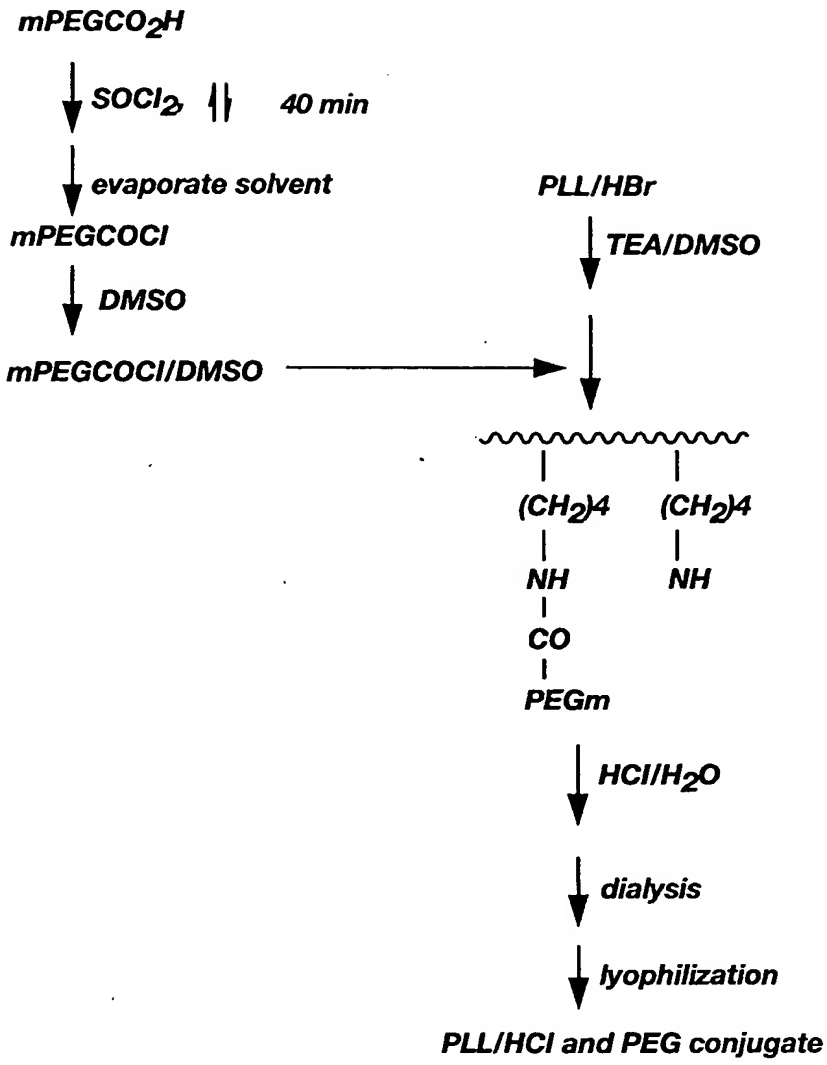
mPEGCO2H
SOCl2
40 min
evaporate solvent
mPEGCOCl
DMSO
mPEGCOCl/DMSO
PLL/HBr
TEA/DMSO
(CH2)4
(CH2)4
NH
NH
CO
PEGm
HCl/H2O
dialysis
lyophilization
PLL/HCl and PEG conjugate

Fig. 2

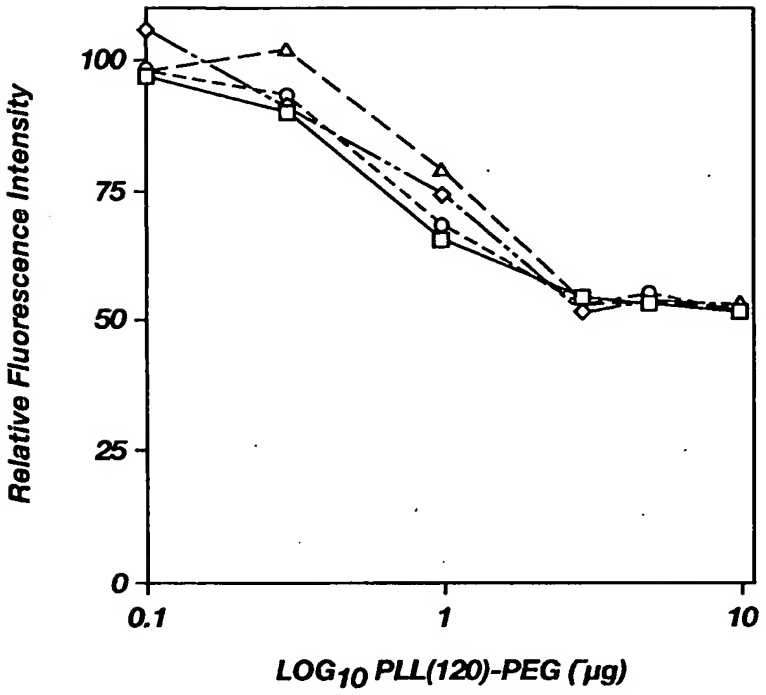
Relative Fluorescence Intensity
100
75
50
25
0
0.1
1
10
LOG10 PLL(120)-PEG (µg)

Fig. 3

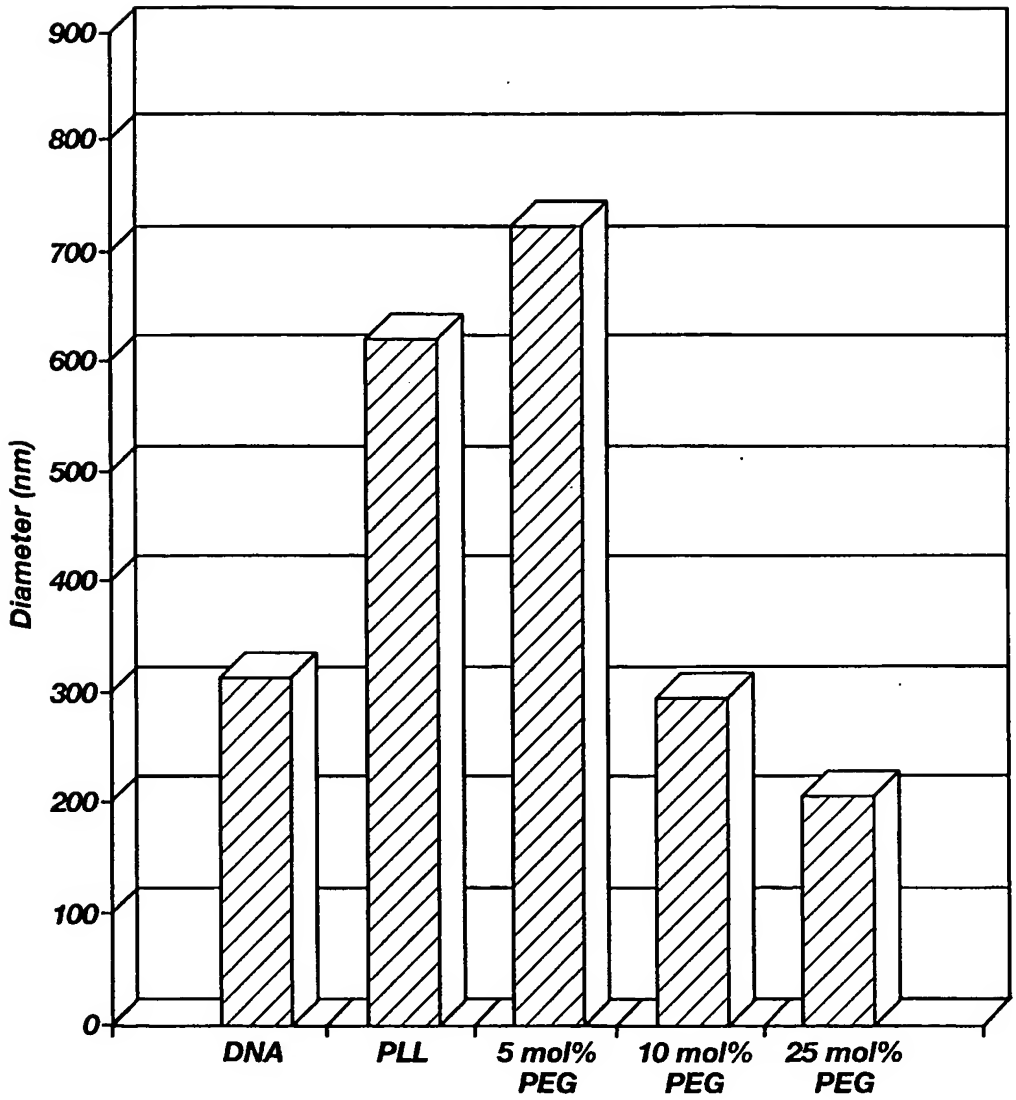
900
800
700
600
500
400
300
200
100
0
Diameter (nm)
DNA
PLL
5 mol% PEG
10 mol% PEG
25 mol% PEG

Fig. 4

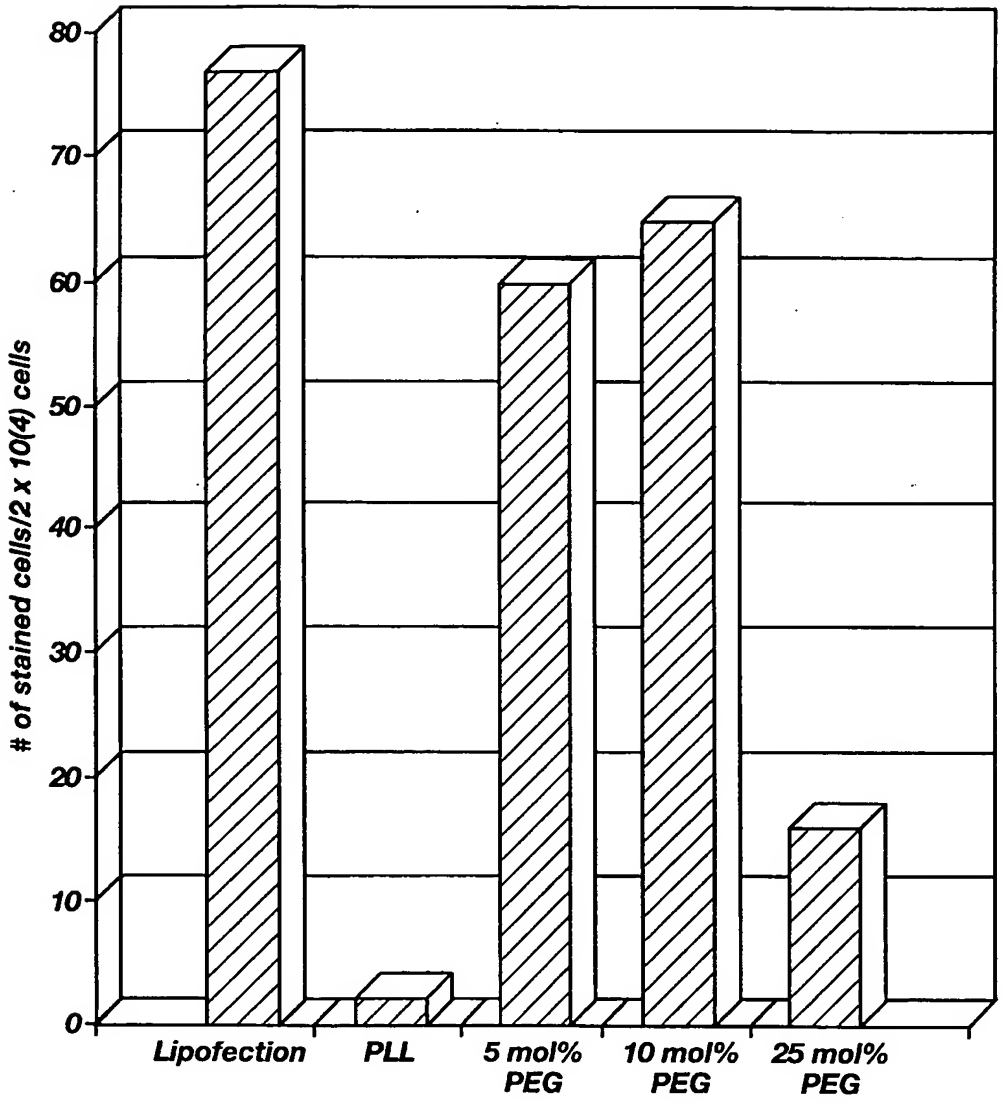
# of stained cells/2 x 10(4) cells
80
70
60
50
40
30
20
10
0
Lipofection
PLL
5 mol% PEG
10 mol% PEG
25 mol% PEG

Fig. 5

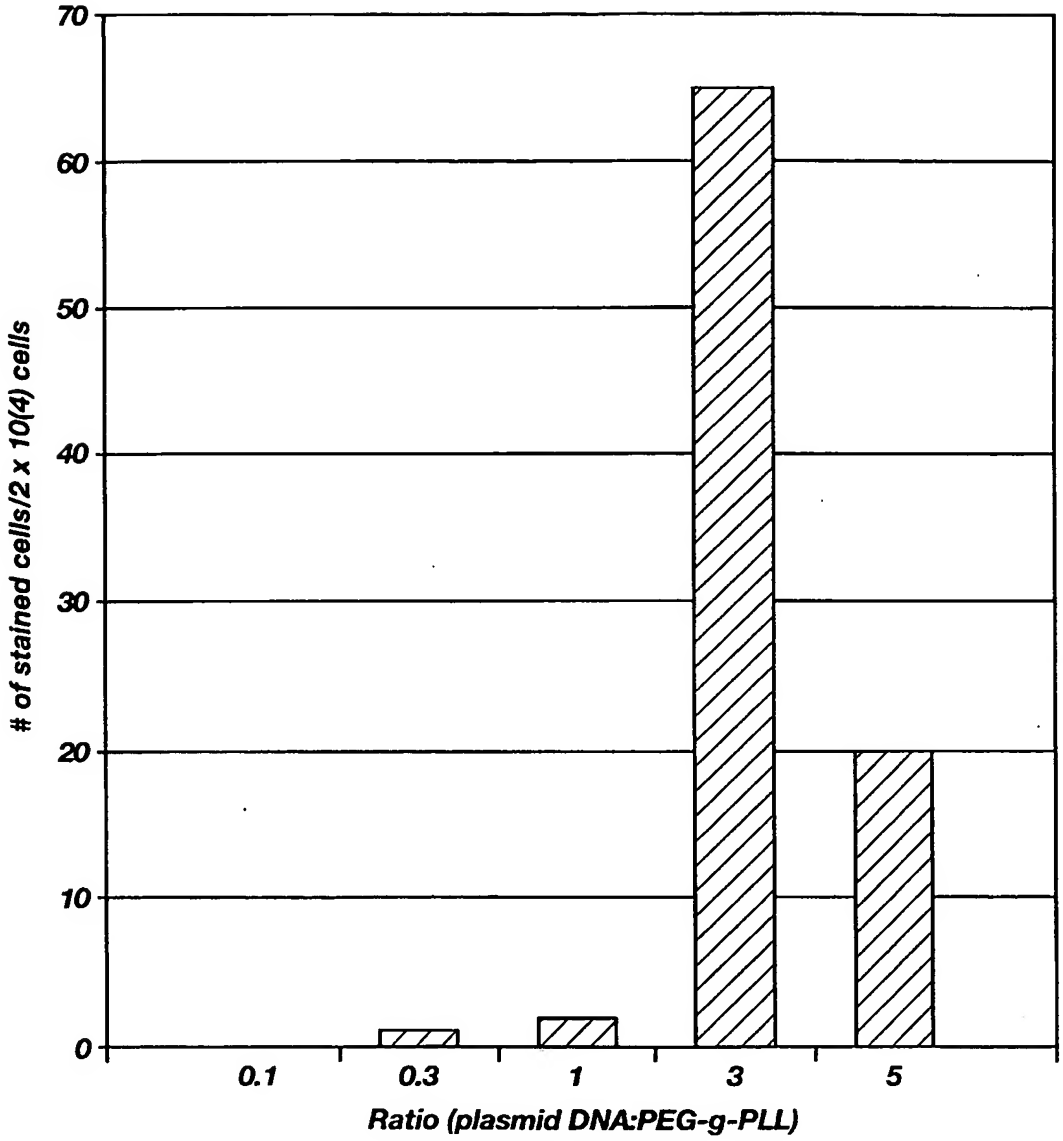
70
60
50
40
30
20
10
0
# of stained cells/2 x 10(4) cells
0.1
0.3
1
3
5
Ratio (plasmid DNA:PEG-g-PLL)

Fig. 6

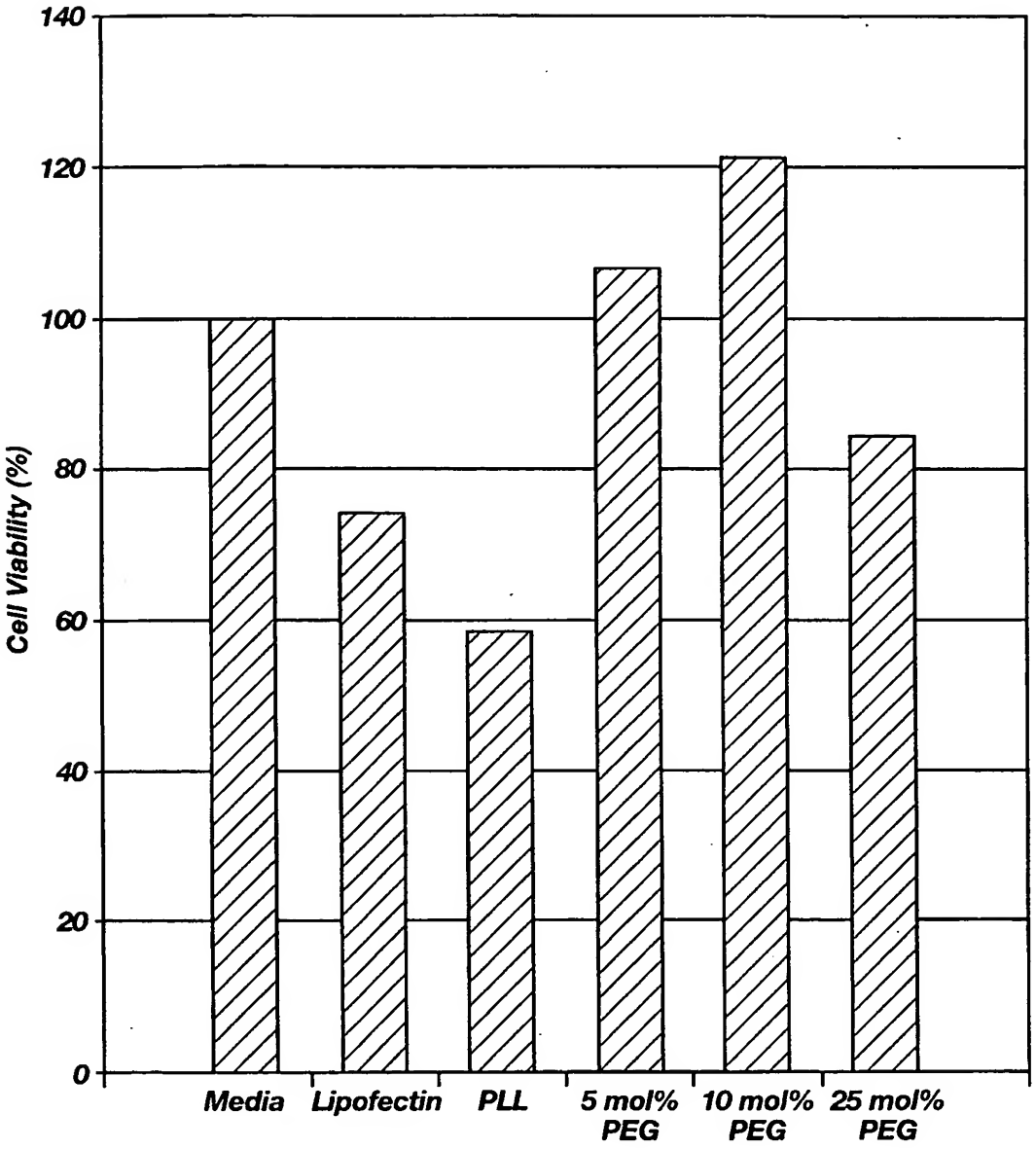
140
120
100
80
60
40
20
0
Cell Viability (%)
Media
Lipofectin
PLL
5 mol% PEG
10 mol% PEG
25 mol% PEG

Fig. 7

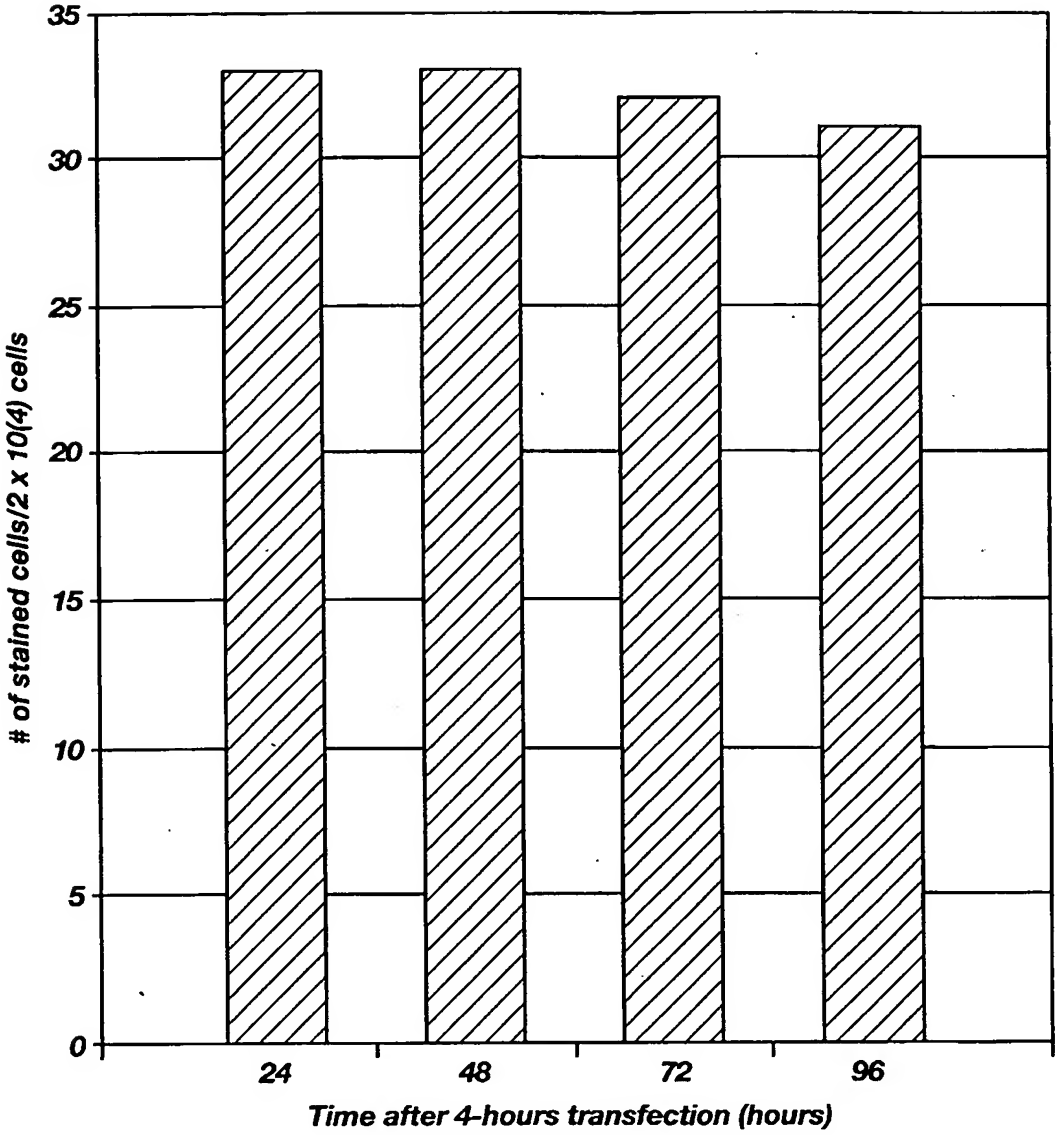
35
30
25
20
15
10
5
0
# of stained cells/2 x 10(4) cells
24
48
72
96
Time after 4-hours transfection (hours)

Fig. 8

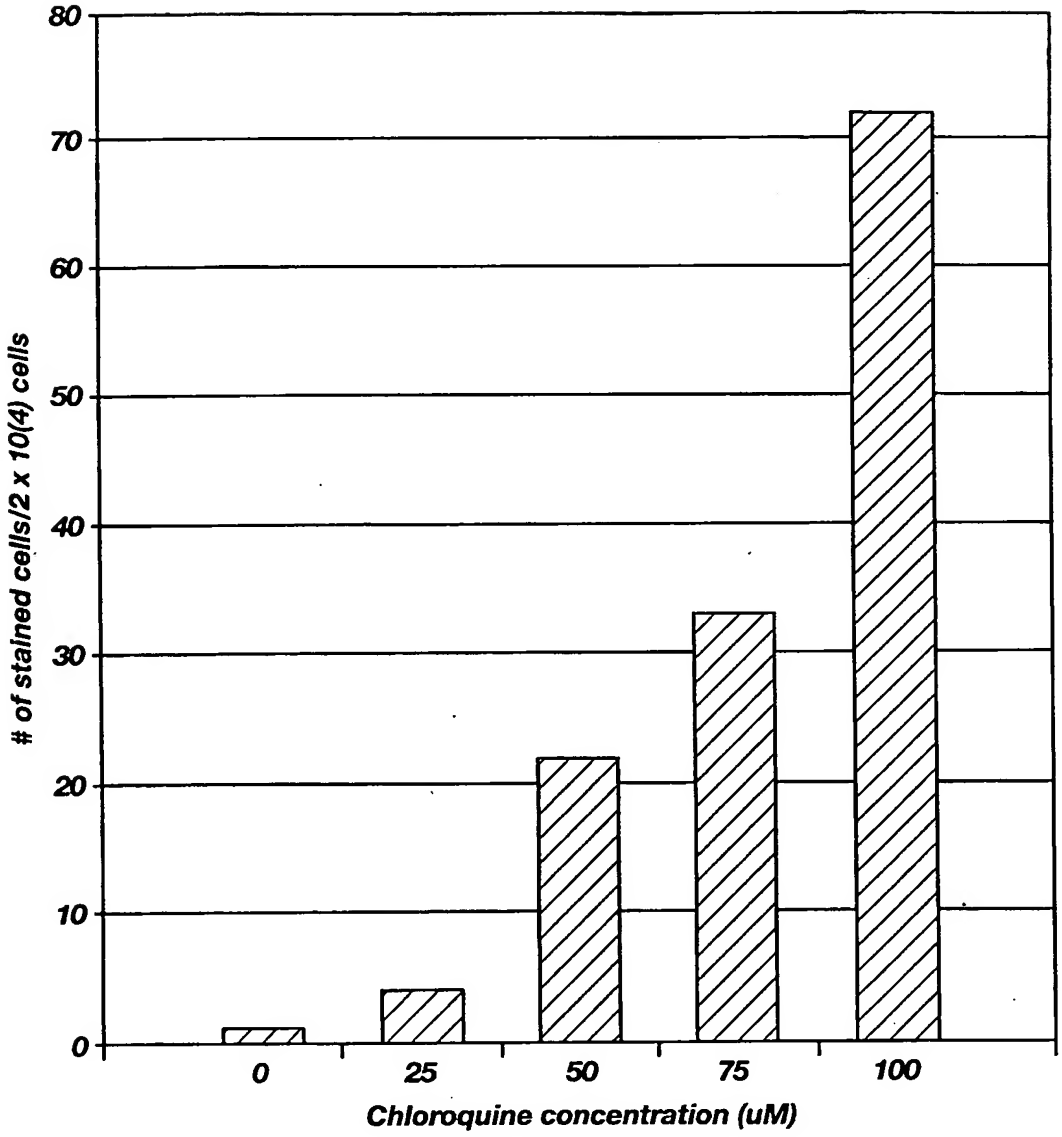
80
70
60
50
40
30
20
10
0
# of stained cells/2 x 10(4) cells
0
25
50
75
100
Chloroquine concentration (uM)

Fig. 9

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26451

A. CLASSIFICATION OF SUBJECT MATTER

IPC(6) :C12N 15/00; A61K 48/00; A01N 63/00
US CL : 435/320.1, 325, 455, 458; 514/44; 424, 93.21
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/320.1, 325, 455, 458; 514/44; 424, 93.21

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

NONE

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

MEDLINE, APS, CAS ONLINE

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database Medline on STN, AN 97467990, KATAYOSE et al. Water-soluble polyion comples associates of DNA and poly(ethylene glycol)-poly(L-lysine) block copolymer. Bioconjugate Chemistry. September-October 1997, Vol. 8, No. 5, pages 702-707, see abstract.	1-3, 5, 6, 9, 10, 13-15, 17, 18, 21, 22, 30-32, , 34, 35, 38, 39, and 47.
X	Database Caplus on STN, AN 1996:489028, KATAYOSE et al. PEG-poly(lysine) block copolymer as a novel type of synthetic gene vector with supramolecular structure. Adv. Biomater. Biomed. Eng. drug Delivery Syst., [Iketani Conf. Biomed. Polym]. 5th (1996). Metting date 1995, pages 319-320, see atstract.	1-3, 5, 6, 9, 10, 13-15, 17, 18, 21, 22, 30-32, 34, 35, 38, 39, and 47.

[X] Further documents are listed in the continuation of Box C. [ ] See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
"E"	earlier document published on or after the international filing date	"Y"	document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
"O"	document referring to an oral disclosure, use, exhibition or other means		
"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 26 FEBRUARY 1999	Date of mailing of the international search report 11 MAR 1999
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer DAVE NGUYEN Telephone No. (703) 308-0196

Form PCT/ISA/210 (second sheet)(July 1992)*

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US98/26451

C (Continuation). DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	Database Embase on STN, AN 96365590, WOLFERT et al. Characterization of vectors for gene therapy formed by self-assembly of DNA with synthetic block co-polymers. Human Gene Therapy. 1996, Vol. 7, No. 17, pages 2123-2133, see abstract.	1-3, 5, 6, 9, 10, 13-15, 17, 18, 21, 22, 30-32, 34, 35, 38, 39, and 47.
X	Database Caplus on STN, KATAYOSE et al. Water soluble polyion complex between DNA and PEG-poly(L-lysine) block copolymer for a novel gene vector . Proc. Int. Symp. controlled Release Bioact. Mater. 1996, Vol. 23, pages 899-900, see abstract.	1-3, 9, 10, 13-15, 21, and 22.
Y	US 5,523,222 A (PAGE et al.) 04 June, 1996, see columns 6-8.	1-47